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Direct assessment of tubuloglomerular feedback responsiveness in connexin 40-deficient mice

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Abstract

Participation of connexin40 (Cx40) in the regulation of renin secretion and in the tubuloglomerular feedback (TGF) component of renal autoregulation suggest that gap junctional coupling through Cx40 contributes to the function of the juxtaglomerular apparatus. In the present experiments we determined the effect of targeted Cx40 deletion in C57Bl/6 and FVB mice on TGF responsiveness. In C57Bl/6 mice, stop flow pressure (P_{SF}) fell from 40.3 ± 2 to 34.5 ± 2 mm Hg in WT and from 31 ± 1.06 to 26.6 ± 0.98 mm Hg in Cx40-/-. P_{SF} changes of 5.85 ± 0.67 Hg in wild type and of 4.3 ± 0.55 mm Hg in Cx40-/- mice were not significantly different (p=0.08). In FVB mice, P_{SF} fell from 36.5 ± 1.8 mm Hg to 30.9 ± 1.75 mm Hg in WT and from 28.1 ± 1.6 to 25.4 ± 1.7 mm Hg in Cx40-/- with mean TGF responses being significantly greater in WT than Cx40-/- (5.5 ± 0.55 mm Hg vs. 2.7 ± 0.84 mm Hg; p=0.002). In both genetic backgrounds P_{SF} values were significantly lower in Cx40-/- than WT mice at all flow rates. Arterial blood pressure in the animals prepared for micropuncture was not different between WT and Cx40-/- mice. We conclude that the TGF response magnitude in superficial cortical nephrons is reduced by 30-50% in mice without Cx40, but that with the exception of a small number of nephrons residual TGF activity is maintained. Thus, gap junctional coupling appears to modulate TGF, perhaps by determining the kinetics of signal transmission.

Key-words: Micropuncture, stop flow pressure, genetic background, vascular resistance

Introduction

Besides being affected by multiple systemic factors, glomerular filtration rate (GFR) is controlled by an intrarenal control mechanism known as tubuloglomerular feedback (TGF). TGF is constructed as a homeostatic feedback loop in which an increase of NaCl concentration in the tubular fluid passing the apical aspect of macula densa cells is translated into a preglomerular vasoconstriction and a concomitant reduction of single nephron GFR. While the relationship between the tubular input and the vascular output is well understood, numerous aspects of the juxtaglomerular transmission pathway are still unclear.

Because of the absence of a structural coupling between macula densa/TAL cells and the underlying mesangium it has been generally assumed that the activation of TGF by elevated tubular NaCl concentrations is accompanied by the generation of paracrine vasoactive factors within the confines of the juxtaglomerular apparatus, and that these paracrine factors mediate the modulation of the afferent arteriolar tone. There is strong experimental evidence in support of the notion that activation of A1 adenosine receptors (A1AR) by NaCl-dependent increases of juxtaglomerular adenosine levels provides the most important vasoconstrictor input with angiotensin II acting as synergistic cofactor. Specific A1AR antagonists such as 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), PSB-36, or KW-3902 markedly attenuate TGF responses, and the genetic ablation of A1AR causes complete loss of TGF function (2, 3, 19, 23, 26). The appearance of adenosine in the JGA interstitium is for the most part the result of dephosphorylation of released ATP by ecto-ATPases and ecto-5'nucleotidase (4, 20). Enhancement of TGF responsiveness by vascular overexpression of A1AR indicates that adenosine may predominantly target A1AR on afferent arterioles without excluding a role of extravascular receptors (17). In fact, selective deletion of A1AR in smooth muscle cells by cre-lox mediated recombination markedly attenuated, but did not abolish TGF responses directly implicating non-vascular A1AR in TGF (13). Nevertheless, the precise location of

adenosine generation and the route by which adenosine reaches vascular or extravascular A1AR is not clear.

In an isolated JGA preparation TGF activation has been shown to cause an increase of cytosolic Ca that spreads across the mesangial cell field and reaches the afferent arteriole (18). Ca propagation was inhibited by heptanol or glycyrrhetinic acid, known inhibitors of gap junctional coupling, suggesting that signal transmission in the JGA may utilize intercellular communication pathways (18). Gap junctions are formed by connexin (Cx) subunits, and several different Cx proteins are expressed in the JGA, including Cx37, Cx40, Cx43, and Cx45 (8). Cx40 is highly expressed in extraglomerular mesangial cells and might therefore be a candidate for the transmission of a depolarization or Ca signal across the JGA (12, 28, 32). Early functional evidence for a role of connexins in the regulation of renal blood flow was provided by the observation that inhibition of connexin formation by connexin-mimetic peptides reduced autoregulatory efficiency (27). The subsequent demonstrations of an impairment of the TGF component of autoregulation in connexin40-deficient mice has directly implicated a specific gap junction protein in the TGF mechanism (9, 25).

In the present experiments, Cx40-deficient mice were used to assess the function of Cx40 in TGF signaling using micropuncture techniques in the intact kidneys of anesthetized mice. Cx40-deficient mice in two different genetic backgrounds exhibited reduced TGF magnitudes and abnormal kinetics, although the degree of TGF impairment was widely variable between nephrons. Nevertheless, our data suggest that Cx40 gap junctions or hemichannels contribute to signal transmission between macula densa cells and the vasculature of the afferent arteriole. On the other hand, maintenance of residual TGF activity in most nephrons of Cx40-deficient mice indicates that macula densa and smooth muscle cells are also connected through pathways that do not require intact Cx40 functionality.

Methods

Animals. To reduce the possible risk of strain-dependent effects, experiments were performed in animals on a C57Bl/6 or FVB genetic background. C57Bl/6 wild type and Cx40-/- mice originally generated by Kirchhoff et al. were obtained from Dr. de Witt (University of Lubeck, Germany) and kept at the animal facility of the University of Regensburg. Animals used were in a weight range between 21 and 28 grams. FVB mice were purchased from Charles River (Sulzfeld, Germany) and crossed with C57Bl/6 Cx40 null mice for at least 4 generations before use (weight range 28 to 37 grams). Genotyping was done according to standard protocols using DNA from tail biopsies. The following gene-specific primers were used: Cx40 forward (ggg aga tga gca ggc cga ctt ccg gtg cg), Cx40 reverse (5'-gta ggg tgc cct gga gga caa tct tcc c-3'), neo forward (5'-gga tcg gcc att gaa caa gat gga ttg cac-3'), and neo reverse (5'-ctg atg ctc ttc gtc cag atc atc ctg atc g-3'). Littermates from heterozygous breeding pairs were used in the experiments. Animals were fed a standard diet and kept at a 12h light/12 h dark cycle.

Animal preparation. For micropuncture experiments mice were anesthetized with 100 mg/kg thiobutabarbital (Inactin®) intraperitoneally and 100 mg/kg ketamine subcutaneously. Body temperature was maintained at 37.5° C by placing the animals on an operating table with a servo-controlled heating plate. The trachea was cannulated, and 100 % oxygen was blown at a low rate towards the tracheal tube throughout the experiment to maintain arterial oxygen saturation. The left carotid artery was catheterized with hand-drawn polyethylene tubing for continuous measurement of arterial blood pressure. A catheter connected to an infusion pump was inserted into the right jugular vein for an intravenous maintenance infusion of saline at 300 µl/hr.

Micropuncture experiments. Measurements of stop flow pressure (P_{SF}) in superficial cortical nephrons during perfusion of the loop of Henle were done as described previously (24, 31). When P_{SF} had stabilized, perfusion rate of the loop of Henle was increased to 30 nl/min and maximum P_{SF} responses were determined.

Perfusion rate was then returned to 0 nl/min. Flow conditions were maintained until steady states were achieved. When possible, two such responses were determined successively in each nephron. The perfusion fluid contained (in mM/L) 136 NaCl, 4 $NaHCO_3$, 4 KCl, 2 $CaCl_2$, 7.5 urea and 100 mg/100 ml FD&C green (Keystone, Bellefonte, PA).

Glomerular filtration rate. GFR in conscious mice was measured by single-injection FITC-inulin or FITC-sinistrin clearance (13). FITC-inulin or FITC-sinistrin (gift from Dr. Schock-Kusch, University of Heidelberg, Germany) was injected at 3.7 µl/g body weight into the retro-orbital plexus during brief sevoflurane anesthesia. At 3, 7, 10, 15, 35, 55, and 75 min, the mice were placed in a restrainer, the tail vein was punctured with a 30-G needle for collection of approximately 2 µl of blood. A total of 500 nl of plasma was then diluted 1:10 in 500 mmol of HEPES (pH 7.4) and measured against a standard curve, as described (7). Fluorescence was determined in 2 µl of the diluted sample (Nanodrop-ND-3300, Nanodrop Technologies, Wilmington, DE). GFR was calculated using a two-compartment model of two-phase exponential decay (7).

Results

Tubuloglomerular feedback. Using animals with the original C57Bl/6 genetic background, useable results were obtained in 7 WT and 7 Cx40-/- mice. Effects of raising loop of Henle flow rate to saturating levels on stop flow pressure (P_{SF}) of individual tubules are summarized in Fig. 1A. On average, P_{SF} fell from 40.3 ± 2 to 34.5 ± 2 mm Hg in WT (n=18) and from 31 ± 1.06 to 26.6 ± 0.98 mm Hg in Cx40-/- mice (n=20). These flow-induced reductions of P_{SF} were highly significant in both WT and Cx40-/- mice ($p<0.001$). The mean difference between P_{SF} at 0 and 30 nl/min, the response magnitude, did not quite achieve significance between genotypes averaging 5.85 ± 0.67 mm Hg in the WT and 4.3 ± 0.55 mm Hg in the Cx40-/- mice ($p=0.08$; Fig. 2A). Failure to achieve significance was the result of considerable variability in the response magnitude in both

163 wild type and Cx40-deficient animals (Fig. 3A). Duration of TGF responses defined as
164 time from perfusion onset to attainment of a steady state was not different between WT
165 and Cx40-/- mice (22.8 ± 3.5 sec vs. 23.9 ± 3 sec). Previous experiments have shown that
166 P_{SF} in response to flow stimulation declines at an initial fast and a subsequent slow rate
167 each contributing about half to the total response magnitude (17). Although differential
168 slope determinations were only possible in a subset of nephrons due to blood pressure
169 instabilities (12 of 18 and 11 of 20 in WT and Cx40-/- mice), the fast and slow slopes of
170 0.87 ± 0.21 and 0.22 ± 0.05 mm Hg/sec in WT are similar to those reported previously
171 (17). Cx40-deficiency reduced or abolished the slope differences by reducing the fast
172 slope (to 0.38 ± 0.07 mm Hg/sec; p=0.017) while the slow slope was not affected (0.19 ±
173 0.04 mm Hg/sec; p=0.66). A P_{SF} reduction to an essentially single slope can be seen in
174 the example of Fig. 4A. P_{SF} values at zero flow rate were consistently and significantly
175 lower in the Cx40-deficient compared to WT mice ($p<0.001$). This observation is
176 indicative of an increased preglomerular vascular resistance in the Cx40-deficient
177 animals since mean arterial blood pressure during micropuncture was similar in WT and
178 Cx40-/- of the C57Bl/6 line (89.8 ± 3.14 mm Hg and 89.4 ± 2.3 mm Hg, respectively).
179 To functionally determine if TGF responsiveness may be modified through availability of
180 A1 adenosine receptors we compared the effect of adding the A1 adenosine receptor
181 agonist cyclohexyl adenosine (CHA) to the perfusate. Perfusion of the loop of Henle with
182 a perfusate containing CHA (10 μM) increased TGF responses in WT to 8.1 ± 0.9 mm
183 Hg (from 5.85 ± 0.66; n=10; $p<0.05$) and to 7.6 ± 0.5 in Cx40-/- (from 4.3 ± 0.55; n=10;
184 $p<0.001$). Cx40-/- mice had a reduction in the fast TGF slope from 0.96 ± 0.18 to 0.28 ±
185 0.05 mm Hg/sec (p=0.003) whereas the slow slopes of 0.28 ± 0.05 and 0.13 ± 0.02 mm
186 Hg/sec were similar ($p>0.05$). Thus, like with standard Ringer perfusion, Cx40-
187 deficiency was associated with a leveling in the slope differential mainly by a marked
188 decrease in the speed of the fast TGF component.

To reduce the possible impact of strain-dependent effects the Cx40 null mutation was bred into an FVB genetic background for more than 4 generations. In these FVB mice successful micropuncture experiments were performed in 6 male WT (mean BW 30 g) and 4 male Cx40-/- (mean BW 31.5 g). P_{SF} decreased with loop perfusion from 36.5 ± 1.8 mm Hg to 30.9 ± 1.75 mm Hg in WT (n=18) whereas it fell from 28.1 ± 1.6 to 25.4 ± 1.7 mm Hg in the FVB Cx40-/- animals (n=14). Data from individual mice are shown in Fig. 1B. Similar to the findings in C57Bl/6 mice, P_{SF} at zero flow was significantly lower in the Cx40-deficient mice compared to WT despite the fact that mean arterial blood pressure during micropuncture tended to be higher in Cx40-/- than WT mice (98.4 ± 2.8 mm Hg vs. 91.3 ± 3.3 mm Hg; p=0.12). Mean TGF response magnitude was 5.5 ± 0.55 mm Hg in the FVB WT and 2.7 ± 0.84 in the FVB Cx40-/- mice (p=0.002; Fig. 2B). Combining the results from both FVB and C57Bl/6 strains reveals a significant reduction of the TGF response magnitude from 5.81 ± 0.41 to 4.12 ± 0.52 mm Hg (p=0.012; Fig. 2C). As in the C57Bl/6 strain, individual TGF responses showed substantial heterogeneity in both WT and Cx40-/- animals with 4 nephrons in the latter group displaying inverted responses (Fig. 3B; Fig. 4B). This difference does not only stem from differences in individual mice, but is also evident in a given mouse suggesting that the response strength is to a large extent a property of the individual tubule. The duration of responses was not different between WT and Cx40-/- mice (37.6 ± 4.9 sec in WT and 44 ± 7.3 sec in Cx40-/- mice). Like C57Bl/6 mice, TGF responses in FVB mice showed a TGF slope differential with a fast slope of 0.72 ± 0.12 mmHg/sec and a slow slope of 0.13 ± 0.02 mmHg/sec (slopes could be determined in 13 of 18 tubules). There was a significant reduction in the fast slope to 0.31 ± 0.06 mm Hg/sec in the FVB Cx40-/- mice (p=0.03; Fig. 4A and 4B) while the slow slope of 0.08 ± 0.01 mm Hg/sec was not significantly different from wild type (slopes could be determined in 7 of 14 tubules). No consistent differences were detected in response onset, the time between changing perfusion rate and the beginning of the P_{SF} decline.

GFR. GFR measured by FITC-inulin plasma clearance kinetics in conscious C57Bl/6 mice averaged 407.5 ± 24 µl/min in wild type (n=12) and 294.2 ± 19 µl/min in Cx40-/- animals (n=12; p=0.0012). Body weights and ages were similar between genotypes (WT: 24.8 ± 1.3 g and 20.3 ± 3.1 wks; KO: 23.9 ± 1.3 g and 18.8 ± 2.3 wks). Using FITC-sinistrin clearance in conscious mice of the FVB background, GFR tended to be lower in Cx40-/- than WT mice without reaching significance (379 ± 96 µl/min vs. 416 ± 53 , p=0.31; n=9). Systolic blood pressure in animals of the FVB background used for GFR measurements was markedly increased in Cx40-deficient mice when compared to wild types (143±7 vs. 124±2 mm Hg, p=0.025, n=5) whereas heart rate was similar in both genotypes, averaging 723±16 and 706±15 beats per minute for Cx40-/- and +/+, respectively (p=0.40; n=5).

Discussion

The results of the present study show that global deletion of connexin 40 (Cx40) attenuates tubuloglomerular feedback (TGF) responses in superficial cortical nephrons of mice, but that TGF responsiveness is not fully abolished in the majority of nephrons. Thus, intercellular communication pathways through Cx40 appear to modulate TGF response magnitude and dynamics without being an absolute requirement for signal transmission.

Connexin 40 is a gap junctional protein that in the kidney is expressed in endothelial cells of most renal vessels, intra-and extraglomerular mesangial cells, and renin-producing juxtaglomerular granular cells (8, 29). The availability of Cx40-deficient mice has permitted a detailed evaluation of the role of this particular gap junction protein in renal function. The observation of dramatic changes in renin cell localization, renin expression, and renin secretion in Cx40-deficient animals has drawn attention to Cx40 as a critical regulator of the function of the juxtaglomerular apparatus (11, 30). Since in addition to regulating renin release the JGA is the site of information transfer from

tubules to afferent arterioles evaluating the role on Cx40 in TGF has been a logical extension of these earlier studies. In this regard, assessments of blood flow autoregulation in intact animals have shown that Cx40-/- mice have a reduced ability to regulate blood flow in response to step blood pressure changes in the time span typical for the TGF component of autoregulation (9). Furthermore, in the perfused juxtamedullary nephron preparation the constrictor response of afferent arterioles to increments of perfusion pressure was blunted in Cx40-deficient mice (25). Prior papillectomy performed to physically disrupt the TGF pathway eliminated the difference between WT and Cx40-/- preparations indicating that a dysfunctional TGF mechanism was mainly responsible for the impairment of autoregulation (25). Nevertheless, autoregulation is a complex response with a number of functional interactions so that a direct assessment of TGF was felt to be a needed addition to the existing evidence.

Disregarding results in singular nephrons our results demonstrate that TGF responses are in general not eliminated in mice with null mutations of Cx40. Furthermore, taken all observations together it also seems well supported that there is a clear attenuation of responses by about 50% in FVB and 30% in C57Bl/6 mice. We realize that these numbers are lower bound estimates of the involvement of gap junctional coupling in TGF since a contribution of other connexins especially of Cx 37 is entirely conceivable. The reasons for the reduced TGF responsiveness is not clear, but it is probably the direct consequence of the absence of Cx40. Our observation that the local administration of the A1AR agonist CHA caused a comparable augmentation of TGF responses in both wild type and Cx40-/- mice makes it unlikely that differences in A1AR expression can account for the different TGF responses. Arterial blood pressure can directly affect TGF responses, but pressure was not different in C57Bl/6 mice without Cx40 and higher in corresponding FVB mice (1, 5, 21). Furthermore, TGF responses are enhanced by angiotensin II, but angiotensin II levels are likely to be higher, not lower in the Cx40-deficient compared to wild type animals (14, 22). An interesting possibility is

that an increase of nitric oxide generated by nNOS in macula densa cells contributes to TGF attenuation since the level of macula densa nNOS has been reported to be upregulated in Cx40-deficient mice (10).

Data in Cx40-deficient mice on both genetic backgrounds show significantly lower levels of stop flow pressure compared to wild type animals regardless of loop of Henle flow rate. These differences are not a reflection of blood pressure since arterial pressure during micropuncture was not different between genotypes in the C57Bl/6 animals and higher in the Cx40-deficient animals on the FVB background. A marked reduction of the baseline diameter of the afferent arteriole close to the vascular pole at constant perfusion pressure has also been observed in juxtamedullary nephron preparations of Cx40-deficient mice (25). It is safe to assume therefore that Cx40-deficiency is associated with an increase of afferent arteriolar resistance near the glomerular entrance. Since stop flow pressure differences were found in the absence of macula densa perfusion, the increased resistance is probably not TGF-mediated. It is possible that the chronically elevated blood pressure in the Cx40-/- mice causes a vascular remodeling providing a structural reason for the increased resistance. However, acute interference with Cx40 or Cx37 functionality by connexin mimetic peptides also caused a reduction of renal blood flow and a large increase of renal vascular resistance (6, 27). The increase of renin release and the presumably elevated levels of angiotensin II associated with Cx40-deficiency could provide another reason for an increased vasomotor tone. Finally, it has been suggested that inhibition of gap junctional coupling eliminates the tonic vasodilatation exerted by an endothelial hyperpolarizing factor that is not nitric oxide or a cyclooxygenase product (6). Whatever the reason for the clearly reduced stop flow pressure in the Cx40-deficient animals, it is noteworthy that filtration was only moderately lower in the Cx40-/- mice, and that previous measurements did not show significant differences in renal blood flow between genotypes (9). Thus,

296 vasodilation of other serial resistances in the kidney, for example in interlobular arteries
297 or efferent arterioles, appear to compensate for the constriction of afferent arterioles.
298 An attempt to interprete the present data is shown in the schematic representation
299 of Fig. 5. This interpretation incorporates the previous demonstration, repeated by two
300 independent groups, that deletion of adenosine 1 receptors (A1AR) completely eliminates
301 TGF responses in mice (2, 26). The most straightforward notion would be that both
302 Cx40-dependent and Cx40-independent components of TGF include a role for A1AR.
303 We propose that interstitial adenosine most likely produced by ATP dephosphorylation
304 interacts with A1AR on mesangial cells and that this triggers afferent constriction by gap
305 junctional transmission, presumably by spreading depolarization and increases of
306 cytosolic calcium (18). Studies in cultured mesangial cells have shown that adenosine
307 increases calcium uptake through a verapamil-sensitive mechanism, and that this effect is
308 mediated by activation of A1AR (15, 16). We assume that this component would be
309 Cx40-dependent and would probably constitute the fast component of TGF. The
310 reduction of the speed of the fast TGF component in Cx40-deficient mice in the present
311 study is consistent with this interpretation. We further propose that adenosine can directly
312 interact with A1AR on smooth muscle cells reaching the vessels by diffusion or
313 generation from ATP near the vasculature. We assume that this component is Cx40-
314 independent, that it is comparably slow, and that it is responsible for the protracted time
315 course of TGF responsiveness. This is consistent with our observation that the duration of
316 TGF-induced changes of stop flow pressure in responding nephrons was not different
317 between genotypes. In addition, such a pathway would almost by definition depend on
318 anatomical characteristics so that structural differences between JGAs of different tubules
319 might explain the marked response variability between different nephrons. It has been
320 postulated earlier that adenosine contributes to TGF through effects independent of gap
321 junctional coupling (27).

In summary, micropuncture determinations of the stop flow pressure response to a saturating flow elevation indicate that a deletion of the gap junctional protein Cx40 reduces TGF responsiveness by about 30%, but that a significant residual TGF activity appears to be Cx40-independent. It is possible that a connexin other than Cx40 may be involved in TGF, but an extracellular transmission pathway could be a viable alternative.

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References

1. **Bell PD, Navar LG.** Stop-flow pressure feedback responses during reduced renal vascular resistance in the dog. *Am J Physiol* 237: F204-209, 1979.

2. **Brown R, Ollerstam A, Johansson B, Skott O, Gebre-Medhin S, Fredholm B, Persson AE.** Abolished tubuloglomerular feedback and increased plasma renin in adenosine A1 receptor-deficient mice. *Am J Physiol Regul Integr Comp Physiol* 281: R1362-1367, 2001.

3. **Carlstrom M, Wilcox CS, Welch WJ.** Adenosine A(2) receptors modulate tubuloglomerular feedback. *Am J Physiol Renal Physiol* 299: F412-417, 2010.

4. **Castrop H, Huang Y, Hashimoto S, Mizel D, Hansen P, Theilig F, Bachmann S, Deng C, Briggs J, Schnermann J.** Impairment of tubuloglomerular feedback regulation of GFR in ecto-5'-nucleotidase/CD73-deficient mice. *J Clin Invest* 114: 634-642, 2004.

5. **Chen YM, Yip KP, Marsh DJ, Holstein-Rathlou NH.** Magnitude of TGF-initiated nephron-nephron interactions is increased in SHR. *Am J Physiol Renal Physiol* 269: F198-204, 1995.

6. **De Vriese AS, Van de Voorde J, Lameire NH.** Effects of connexin-mimetic peptides on nitric oxide synthase- and cyclooxygenase-independent renal vasodilation. *Kidney Int* 61: 177-185, 2002.

7. **Faulhaber-Walter R, Chen L, Oppermann M, Kim SM, Huang Y, Hiramatsu N, Mizel D, Kajiyama H, Zerfas P, Briggs JP, Kopp JB, Schnermann J.** Lack of A1 adenosine receptors augments diabetic hyperfiltration and glomerular injury. *J Am Soc Nephrol* 19: 722-730, 2008.

8. **Hanner F, Sorensen CM, Holstein-Rathlou NH, Peti-Peterdi J.** Connexins and the kidney. *Am J Physiol Regul Integr Comp Physiol* 298: R1143-1155, 2010.

9. **Just A, Kurtz L, de Wit C, Wagner C, Kurtz A, Arendshorst WJ.** Connexin 40 mediates the tubuloglomerular feedback contribution to renal blood flow autoregulation. *J Am Soc Nephrol* 20: 1577-1585, 2009.

10. **Krattinger N, Alonso F, Capponi A, Mazzolai L, Nicod P, Meda P, Haefliger JA.** Increased expression of renal cyclooxygenase-2 and neuronal nitric oxide synthase in hypertensive Cx40-deficient mice. *J Vasc Res* 46: 188-198, 2009.

11. **Krattinger N, Capponi A, Mazzolai L, Aubert JF, Caille D, Nicod P, Waeber G, Meda P, Haefliger JA.** Connexin40 regulates renin production and blood pressure. *Kidney Int* 72: 814-822, 2007.

12. **Kurtz L, Madsen K, Kurt B, Jensen BL, Walter S, Banas B, Wagner C, Kurtz A.** High-level connexin expression in the human juxtaglomerular apparatus. *Nephron Physiol* 116: p1-8, 2010.

13. **Li L, Lai EY, Huang YG, Eisner C, Mizel D, Wilcox CS, Schnermann JB.** Renal afferent arteriolar and tubuloglomerular feedback reactivity in mice with conditional deletions of adenosine 1 receptors. *Am J Physiol Renal Physiol* 303: F1166-1175, 2012.

14. **Mitchell KD, Navar LG.** Enhanced tubuloglomerular feedback during peritubular infusions of angiotensins I and II. *Am J Physiol Renal Physiol* 255: F383-390, 1988.

15. **Olivera A, Lopez-Rivas A, Lopez-Novoa JM.** Adenosine stimulates Ca2+ fluxes and increases cytosolic free Ca2+ in cultured rat mesangial cells. *Biochem J* 282 (Pt 3): 871-876, 1992.

16. **Olivera A, Tomas M, Lopez-Novoa JM.** Effect of adenosine A1 and A2 agonists and antagonists on cAMP and Ca2+ in cultured rat mesangial cells. *Am J Physiol* 262: C840-844, 1992.

17. **Oppermann M, Qin Y, Lai EY, Eisner C, Li L, Huang Y, Mizel D, Fryc J, Wilcox CS, Briggs J, Schnermann J, Castrop H.** Enhanced tubuloglomerular feedback in mice with vascular overexpression of A1 adenosine receptors. *Am J Physiol Renal Physiol* 297: F1256-1264, 2009.

18. **Peti-Peterdi J.** Calcium wave of tubuloglomerular feedback. *Am J Physiol Renal Physiol* 291: F473-480, 2006.

19. **Ren Y, Arima S, Carretero OA, Ito S.** Possible role of adenosine in macula densa control of glomerular hemodynamics. *Kidney Int* 61: 169-176, 2002.

20. **Ren Y, Garvin JL, Liu R, Carretero OA.** Role of macula densa adenosine triphosphate (ATP) in tubuloglomerular feedback. *Kidney Int* 66: 1479-1485, 2004.

21. **Schnermann J, Briggs JP.** Interaction between loop of Henle flow and arterial pressure as determinants of glomerular pressure. *Am J Physiol Renal Physiol* 256: F421-F429, 1989.

22. **Schnermann J, Briggs JP.** Single nephron comparison of the effect of loop of Henle flow on filtration rate and pressure in control and angiotensin II-infused rats. *Miner Electrolyte Metab* 15: 103-107, 1989.

23. **Schnermann J, Weihprecht H, Briggs JP.** Inhibition of tubuloglomerular feedback during adenosine1 receptor blockade. *Am J Physiol Renal Physiol* 258: F553-561, 1990.

24. **Schnermann JB, Traynor T, Yang T, Huang YG, Oliverio MI, Coffman T, Briggs JP.** Absence of tubuloglomerular feedback responses in AT1A receptor- deficient mice. *Am J Physiol Renal Physiol* 273: F315-320, 1997.

25. **Sorensen CM, Giese I, Braunstein TH, Brasen JC, Salomonsson M, Holstein-Rathlou NH.** Role of connexin40 in the autoregulatory response of the afferent arteriole. *Am J Physiol Renal Physiol* 303: F855-863, 2012.

26. **Sun D, Samuelson LC, Yang T, Huang Y, Paliege A, Saunders T, Briggs J, Schnermann J.** Mediation of tubuloglomerular feedback by adenosine: Evidence from mice lacking adenosine 1 receptors. *Proc Natl Acad Sci U S A* 98: 9983-9988., 2001.

27. **Takenaka T, Inoue T, Kanno Y, Okada H, Hill CE, Suzuki H.** Connexins 37 and 40 transduce purinergic signals mediating renal autoregulation. *Am J Physiol Regul Integr Comp Physiol* 294: R1-11, 2008.

28. **Takenaka T, Inoue T, Kanno Y, Okada H, Meaney KR, Hill CE, Suzuki H.** Expression and role of connexins in the rat renal vasculature. *Kidney Int* 73: 415-422, 2008.

29. **Wagner C.** Function of connexins in the renal circulation. *Kidney Int* 73: 547-555, 2008.

30. **Wagner C, de Wit C, Kurtz L, Grunberger C, Kurtz A, Schweda F.** Connexin40 is essential for the pressure control of renin synthesis and secretion. *Circ Res* 100: 556-563, 2007.

31. **Wright FS, Schnermann J.** Interference with feedback control of glomerular filtration rate by furosemide, triflocin, and cyanide. *J Clin Invest* 53: 1695-1708, 1974.

32. **Zhang J, Hill CE.** Differential connexin expression in preglomerular and postglomerular vasculature: accentuation during diabetes. *Kidney Int* 68: 1171-1185, 2005.

492 Figure Legends

493

494 Fig. 1 Tubuloglomerular feedback responses of tubular stop flow pressure (P_{SF}) in wild
495 type control (WT) and connexin 40-deficient mice (Cx40-/-). A: C57Bl/6 genetic
496 background; B: FVB genetic background. Lines connect data from individual
497 nephrons at perfusion rates of 0, 30, and 0 nl/min. P values are given for
498 comparisons with the 30 nl/min perfusion rate (paired t-test)

499

500 Fig. 2 Reduction of tubular stop flow pressure (P_{SF}) in response to a flow increase from
501 0 to 30 nl/min in WT and Cx40-/- mice on a C57Bl/6 (A) or FVB (B) genetic
502 background, and in both strains combined (C). Data are means ± SEM. Statistical
503 comparison by t-test.

504

505 Fig. 3 TGF responses in individual nephrons of wild type (+/+) and Cx40-deficient mice
506 (-/-) on C57Bl/6 (left) and FVB (right) genetic backgrounds. A: TGF responses
507 magnitude in individual nephrons; B: TGF response magnitude in individual mice
508 (averages of 1-4 nephrons per mouse). Mean values are indicated by horizontal
509 lines.

510

511 Fig. 4 Original recordings of TGF responses of stop flow pressure (P_{SF}) in wild type
512 (Cx40+/+) and connexin40-deficient mice (Cx40-/-). A: Recordings in two
513 nephrons of C57Bl/6 mice; arterial blood pressure (MAP) recordings are shown
514 above the stop flow pressure traces (upper:wild type, lower Cx40-/-). Periods of
515 loop perfusion at 30 nl/min are indicated by grey bars; onset and offset of
516 perfusion is also indicated by dotted lines. Red lines highlight initial declines of
517 P_{SF}. B: Recording in 3 nephrons of FVB mice; traces show arterial blood pressure
518 above P_{SF} (upper: wild type, middle: Cx40-/-, bottom: Cx40-/-); note steep initial

519 fall of P_{SF} as indicated by red line in WT and a markedly slower initial P_{SF} slope
520 in Cx40-/-. Absence of TGF responses as shown in the third example of this graph
521 was seen in a small number of nephrons.
522 Fig. 5 Schematic representation of the proposed role of connexin40 (red boxes) in the
523 TGF response. A Cx40-dependent pathway linking mesangial cells (grey ovals)
524 with the arteriolar smooth muscle cells (brown) is paralled by a Cx40-
525 independent pathway permitting direct paracrine smooth muscle cell activation.
526 Both pathways are assumed to be activated by A1AR based on the previous
527 demonstration of an absolute requirement of A1AR for TGF responsiveness (2,
528 25).
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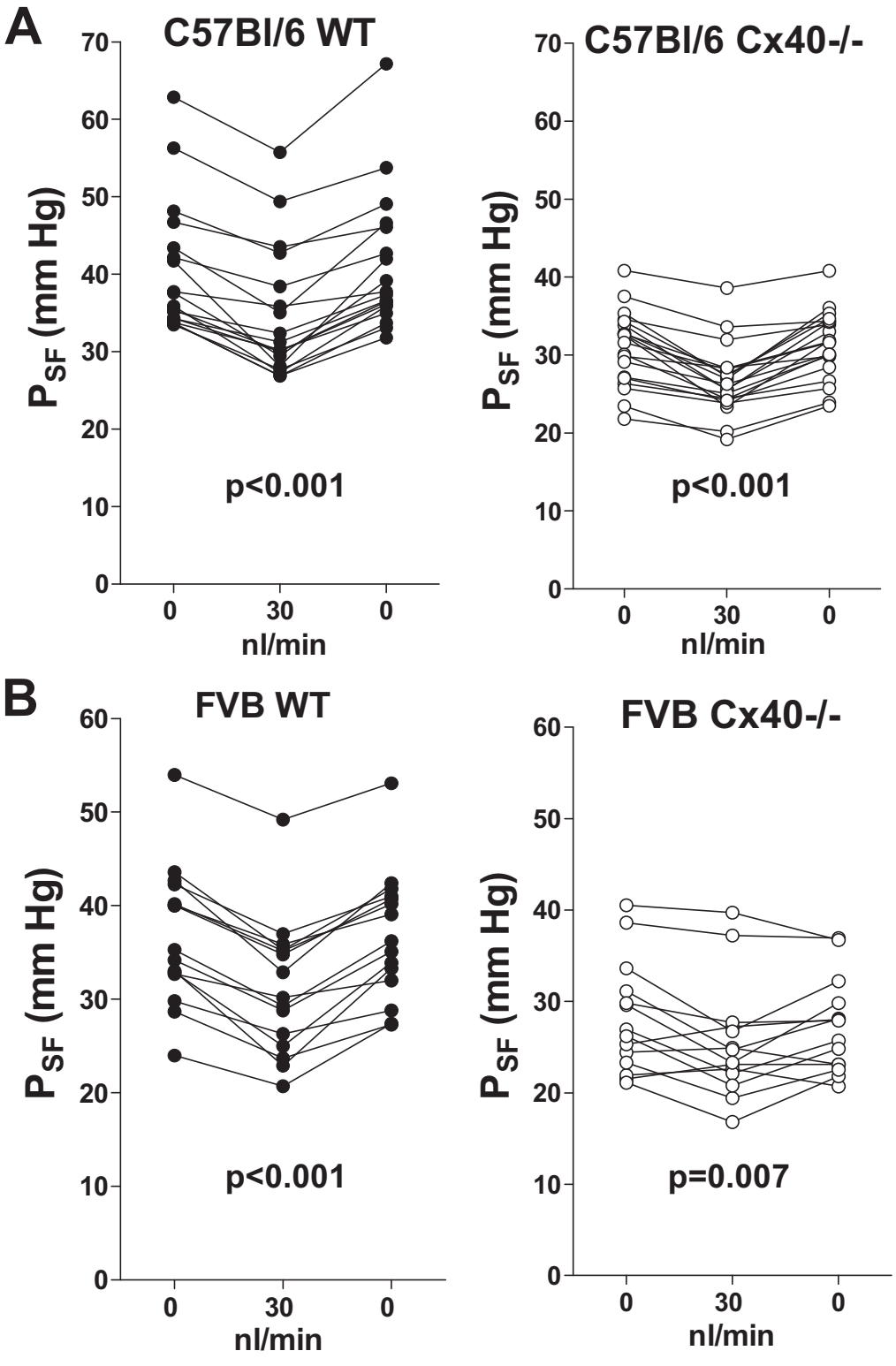
A
C57Bl/6 WT
C57Bl/6 Cx40-/-
P_{SF} (mm Hg)
p<0.001
p<0.001
0
30
0
nl/min
B
FVB WT
FVB Cx40-/-
p<0.001
p=0.007

Fig. 1

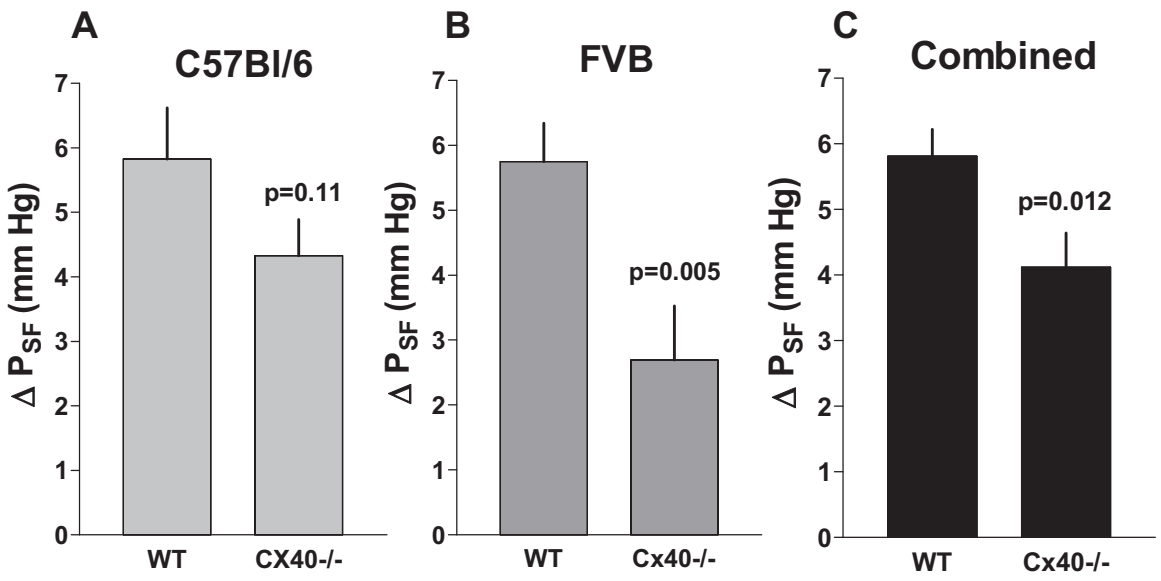
A
C57Bl/6
B
FVB
C
Combined
Δ P_SF (mm Hg)
0
1
2
3
4
5
6
7
p=0.11
p=0.005
p=0.012
WT
CX40-/-
Cx40-/-

Fig. 2

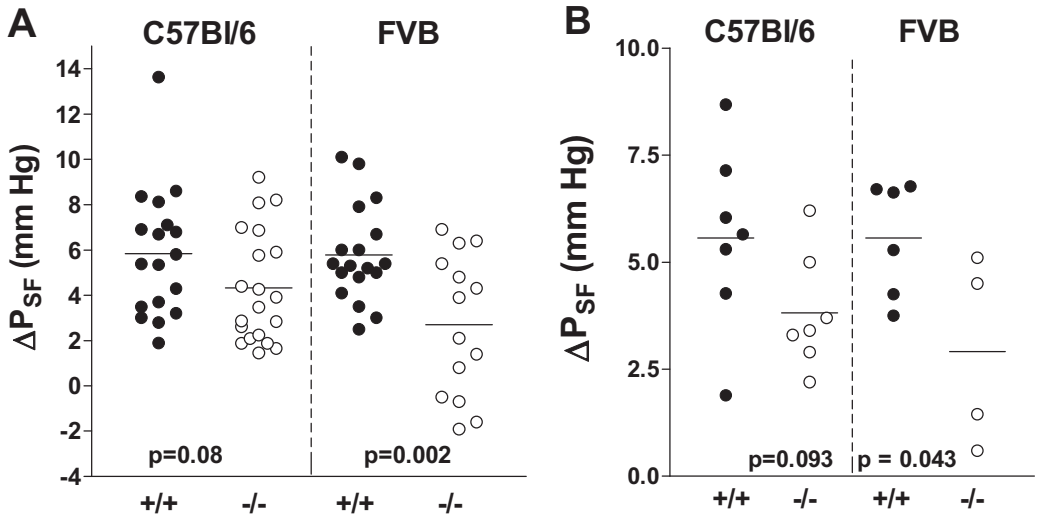
A
C57Bl/6
FVB
ΔP_SF (mm Hg)
14
12
10
8
6
4
2
0
-2
-4
p=0.08
p=0.002
+/+
-/-
+/+
-/-
B
C57Bl/6
FVB
ΔP_SF (mm Hg)
10.0
7.5
5.0
2.5
0.0
p=0.093
p = 0.043
+/+
-/-
+/+
-/-

Fig. 3

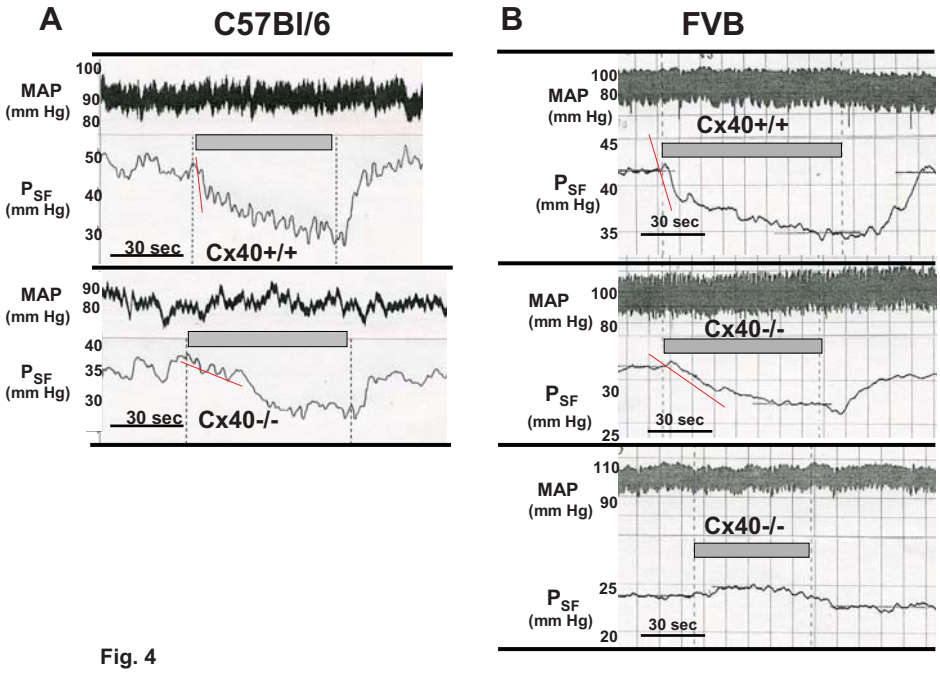
A
C57Bl/6
MAP
(mm Hg)
100
90
80
P_{SF}
(mm Hg)
50
40
30
30 sec
Cx40+/+
MAP
(mm Hg)
90
80
P_{SF}
(mm Hg)
40
35
30
30 sec
Cx40-/-
B
FVB
MAP
(mm Hg)
100
80
Cx40+/+
P_{SF}
(mm Hg)
45
40
35
30 sec
MAP
(mm Hg)
100
80
Cx40-/-
P_{SF}
(mm Hg)
30
25
30 sec
MAP
(mm Hg)
110
90
Cx40-/-
P_{SF}
(mm Hg)
25
20
30 sec

Fig. 4

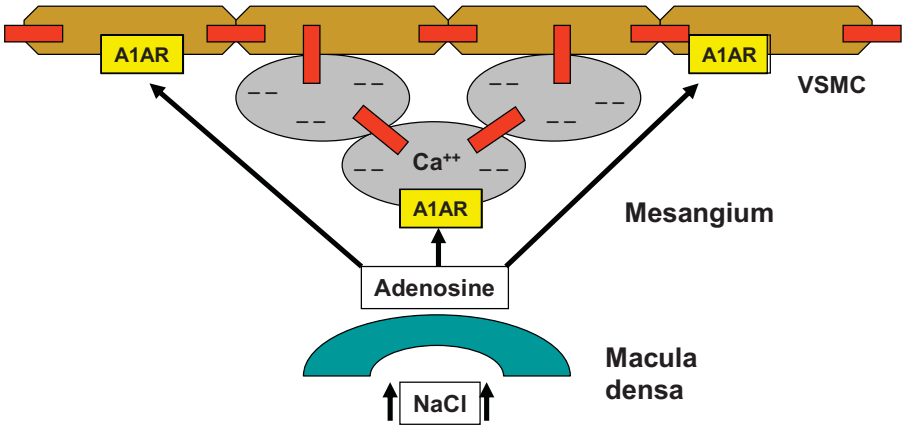
A1AR
A1AR
VSMC
Ca++
A1AR
Mesangium
Adenosine
Macula
densa
NaCl

Fig. 5